

## Temporal Variability of Coastal *Planctomycetes* Clades at Kabeltonne Station, North Sea<sup>∇†</sup>

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Members of the bacterial phylum *Planctomycetes* are reported in marine water samples worldwide, but quantitative information is scarce. Here we investigated the phylogenetic diversity, abundance, and distribution of *Planctomycetes* in surface waters off the German North Sea island Helgoland during different seasons by 16S rRNA gene analysis and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). Generally *Planctomycetes* are more abundant in samples collected in summer and autumn than in samples collected in winter and spring. Statistical analysis revealed that *Planctomycetes* abundance was correlated to the *Centrales* diatom bloom in spring 2007. The analysis of size-fractionated seawater samples and of macroaggregates showed that ~90% of the *Planctomycetes* reside in the >3- $\mu$ m size fraction. Comparative sequence analysis of 184 almost full-length 16S rRNA genes revealed three dominant clades. The clades, named *Planctomyces*-related group A, uncultured *Planctomycetes* group B, and *Pirellula*-related group D, were monitored by CARD-FISH using newly developed oligonucleotide probes. All three clades showed recurrent abundance patterns during two annual sampling campaigns. Uncultured *Planctomycetes* group B was most abundant in autumn samples, while *Planctomyces*-related group A was present in high numbers only during late autumn and winter. The levels of *Pirellula*-related group D were more constant throughout the year, with elevated counts in summer. Our analyses suggest that the seasonal succession of the *Planctomycetes* is correlated with algal blooms. We hypothesize that the niche partitioning of the different clades might be caused by their algal substrates.

*Planctomycetes* are not planktonic fungi but a monophyletic group of *Bacteria* drawing increasing interest for microbial ecologists as well as evolutionary and cell biologists (12). On the basis of comparative 16S rRNA gene sequence analysis, this independent phylum belongs to the so-called *Planctomyces-Verrucomicrobia-Chlamydiae* (PVC) superphylum (55) that also includes the phylum *Lentisphaerae*, the candidate phylum *Poribacteria* (10), and the uncultured clade OP3. The grouping of these phyla into one superphylum is supported by unique features that distinguish its members from other members of the domain *Bacteria*, such as a peptidoglycan-free proteinaceous cell wall (25, 31), cellular compartmentalization (32, 33) and budding type of reproduction (12). Furthermore, *Planctomycetes* cells are often organized in a polar manner and attach to surfaces by nonprosthecate appendages (stalks) or holdfast structures.

Although the first microscopic observation of rosette-forming “planktonic mycetes” was reported back in 1924 (58), pure cultures were not obtained until 1973 (51). Most strains have been isolated from marine, freshwater, and terrestrial habitats (2, 11, 13, 14, 26–28, 48, 57). Despite the recent success in isolating new members of the *Planctomycetaceae* (60), the phy-

lum remains underrepresented in microbial culture collections. The *Planctomycetes* phylum is currently comprised of only two classes, *Planctomycetacia* and *Phycisphaerae*. The *Planctomycetacia* today consists of one order (*Planctomycetales*) and one family (*Planctomycetaceae*) with nine accepted genera (*Planctomyces*, *Pirellula*, *Blastopirellula*, *Rhodopirellula*, *Gemmata*, *Isosphaera*, *Singulisphaera*, *Schlesneria*, and *Zavarzinella*) (26–28, 49, 58). The class *Planctomycetacia* also contains five candidate genera (*Candidatus* Kuenenia, *Candidatus* Brocadia, *Candidatus* Scalindua, *Candidatus* Anammoxoglobus, and *Candidatus* Jettenia). *Phycisphaerae* is a recently accepted new class of *Planctomycetes* (13) composed of a single species, *Phycisphaera mikurensis*, isolated from the marine alga *Porphyra* sp.

By using cultivation-independent methods like 16S rRNA gene sequencing and fluorescence *in situ* hybridization (FISH), *Planctomycetes* were detected in the marine water column (8, 54); they were often found colonizing marine snow particles (7, 8) and were found in marine sediments (34, 38, 46) and in freshwater lakes (22, 23, 36, 39, 57). Several studies have reported high numbers of *Planctomycetes* in response to algal blooms (3, 9, 21, 53, 58). Morris and coworkers (37) have found a peak of *Pirellula*-related cells (up to  $4 \times 10^4$  ml<sup>-1</sup>) during a diatom bloom in Oregon coastal waters. This is consistent with a chemoorganoheterotrophic metabolism of marine *Planctomycetes* and genome annotations. The coastal strain *Rhodopirellula baltica* contains a high number of carbohydrate-active enzymes, including many sulfatases (16). These are known to be involved in the initial breakdown of sulfated heteropolysac-

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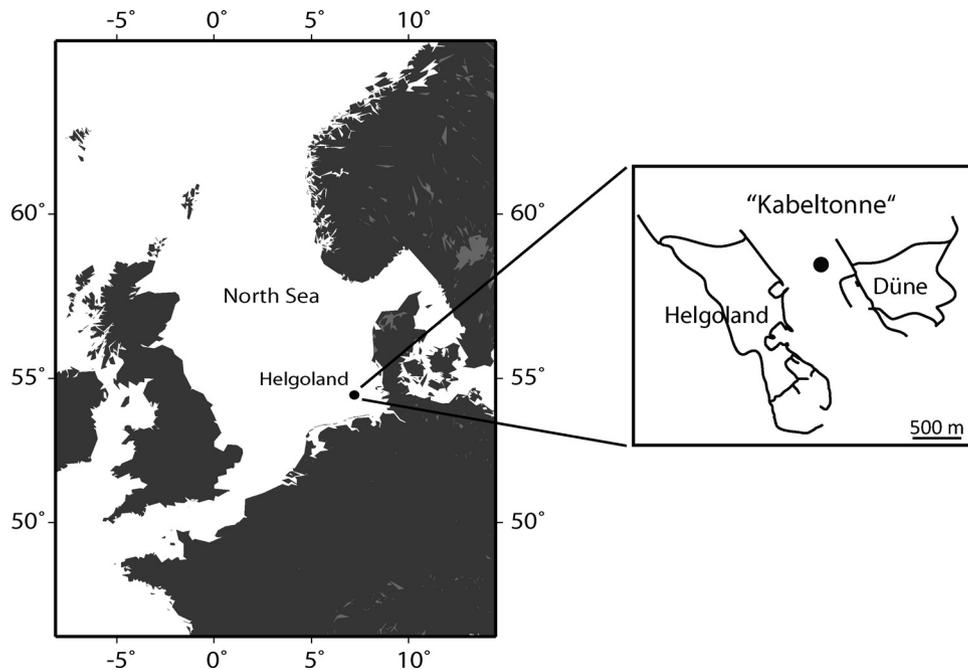


FIG. 1. Location of Helgoland island in the North Sea. The map was created with an online map creation tool and modified manually (<http://woodshole.er.usgs.gov/mapit/>).

charides like carrageenans produced by macroalgae. Recently, a comparison of five available *Planctomycetes* genomes with six *Planctomycetes* fosmids from two marine upwelling systems corroborated the specialization of marine *Planctomycetaceae* for algal polymer degradation (62).

Despite their role in marine carbon cycling, little is known about the diversity of *Planctomycetes*, and particularly, quantitative information on the spatial and temporal distribution of specific planctomycetal clades is scarce. The aim of this study was an assessment of the diversity, abundance, and distribution of planktonic *Planctomycetes* at the coastal marine station Kabeltonne off the Helgoland island in the German Bight of the North Sea (Fig. 1). First, the temporal variability of *Planctomycetes* in relation to the abiotic and biotic parameters in the North Sea was examined in samples from 2007. A size fractionation of these samples was used to clarify the preferred lifestyle of *Planctomycetes* in the water column at Helgoland. Second, dominant *Planctomycetes* clades were identified by comparative 16S rRNA gene analysis and quantified by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) in samples from two annual sampling campaigns in 2007 and 2009.

#### MATERIALS AND METHODS

**Sample collection and abiotic and biotic parameters.** Twenty-three water samples were collected between February and October 2007 and 13 samples were collected between February and November 2009 at a depth of 1 m at the Kabeltonne station (54°11.3'N, 7°54.0'E), Helgoland Roads, North Sea by the research boat *Aade* (Fig. 1). Marine macroaggregates were collected by scuba diving using open-ended syringes on 5 June 2007 from the same sampling site. Determination of salinity was performed using an inductive salinometer (GDT Autosal8400 salinometer; Guildline, Ontario, Canada) followed by conversion to a salinity value using United Nations Educational, Scientific and Cultural Organization (UNESCO) tables (6, 20). The concentrations of silicate, phosphate, nitrate, nitrite, and ammonium were measured photometrically as described

previously (19, 20). For the enumeration of diatoms, samples were preserved with Lugol's solution and counted using the Uthermühl method and an inverted microscope (59) (see Table S1 in the supplemental material).

**Filtration and size fractionation in 2007.** For catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH), water samples were collected and fixed for 1 h at room temperature and up to 24 h at 4°C with paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA; final concentration of 1%). Aliquots from samples collected in winter and autumn were directly filtered onto 0.2- $\mu$ m polycarbonate filters (47-mm diameter; Millipore, Eschborn, Germany). In order to remove resuspended sediment particles, samples from May, June, and July 2007 were first prefiltered through 10- $\mu$ m-pore-size polycarbonate filter (Millipore, Eschborn, Germany). The filtrate was then filtered onto a 0.2- $\mu$ m-pore-size polycarbonate filter (Millipore, Eschborn, Germany) (the 0.2- to 10- $\mu$ m fraction). Additionally, parallel samples were pre-screened with a 3- $\mu$ m-pore-size polycarbonate filter to remove all small aggregates, and attached living cells on microalgae or transparent exopolymer particles (TEP) (the 0.2- to 3- $\mu$ m fraction). All aliquots were 15 ml and were filtered at low pressure (200 mbar). All filters were stored at -20°C until further processing. The abundance of *Planctomycetes* was always quantified on the 0.2- $\mu$ m filters. In 2009, 10-ml and 100-ml sample aliquots were directly filtered onto 0.2- $\mu$ m-pore-size polycarbonate filters (47-mm diameter) without pre-screening.

Macroaggregates were allowed to sediment, resuspended in sterile seawater, fixed, and sonicated on ice (21 W for 60 s) (Sonopuls HD 60; Bandelin, Berlin, Germany). Aliquots of 15 ml were first prefiltered through 10- $\mu$ m-pore-size polycarbonate filters to remove the algae and subsequently filtered onto 0.2- $\mu$ m-pore-size polycarbonate filters.

**CARD-FISH.** Prior to CARD-FISH, the filters were cut in sections and embedded in 0.2% (wt/vol) agarose (gel strength  $\geq 300$  g cm<sup>-2</sup>; Biozym, Oldendorf, Germany) (41). The efficiency of the permeabilization treatments of the *Planctomycetes* cell wall for CARD-FISH was checked using lysozyme, achromopeptidase, and proteinase K. The highest numbers of *Planctomycetes* hybridized by CARD-FISH were obtained after incubation with lysozyme (10 mg ml<sup>-1</sup>; Fluka, Steinheim, Germany; buffer contained 0.05 M EDTA [pH 8] and 0.1 M Tris-HCl [pH 7.4]) for 1 h at 37°C followed by achromopeptidase (60 U ml<sup>-1</sup>; Sigma, Steinheim, Germany; buffer contained 0.01 M NaCl and 0.01 M Tris-HCl [pH 8.0]) for 30 min at 37°C.

Endogenous peroxidases were inactivated by using 0.01 M HCl at room temperature for 10 min. Hybridization with horseradish peroxidase (HRP)-labeled oligonucleotide probes (Table 1; Biomers, Ulm, Germany) and tyramide signal

TABLE 1. *Planctomyces*-specific oligonucleotide probes

Probe	Target organism	Sequence (5' → 3')	FA concn <sup>a</sup> (%)	Reference
PLA46	<i>Planctomyces</i>	GACTTGCATGCCTAATCC	30	39
PirD1039	<i>Pirellula</i> -related group D	ATGCAGCACCTACGTAAG	30	This study
uPlaB440	Uncultured <i>Planctomyces</i> group B	TGCATTTCTCCCAACG	30	This study
PlaA1228	<i>Planctomyces</i> -related group A	AATACCACGCAGACCGCT	30	This study

<sup>a</sup> Formamide (FA) concentration (vol/vol) in CARD-FISH hybridization buffer.

amplification were carried out by the protocol of Pernthaler and colleagues (42) with the following modifications: hybridization was performed at 46°C for 2.5 h, and the washing step was conducted at 48°C for 5 min. The probe-delivered horseradish peroxidase was detected with tyramides labeled with fluorescein (42). The probe NON338 (56) was used as a negative control. No false-positive signals were detected in the samples. After amplification, filter sections were washed twice in 96% ethanol before drying. Filter sections were then counterstained with DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 1 µg ml<sup>-1</sup> and washed for 1 min in Milli-Q water and 96% ethanol. Filter sections were arranged on microscope slides, and embedded in an antifading reagent (Vectashield mounting medium H-1000; Vector Laboratories, Inc., Burlingame, CA, and CitiFluor Ltd., London, United Kingdom). The slides were stored at -20°C until further analyses. Cells were quantified with an Axioplan II imaging epifluorescence microscope (Zeiss, Jena, Germany).

The CARD-FISH signals were strong, and the background was almost black, resulting in a high signal-to-noise ratio. Reddish autofluorescence from cyanobacteria and algae could be easily discriminated from the green CARD-FISH signals. A minimum of 20 microscopic fields were counted comprising at least 1,000 DAPI-stained cells. Clade-specific counts were done on high-density filters on which 100 ml of surface water had been filtered. This allowed for reliable quantifications of populations as small as 0.1% (17). DAPI counts of total picoplankton were determined on untreated filter sections.

**DNA isolation and PCR amplification.** For DNA isolation, approximately 1 liter of water was filtered onto 0.2-µm-pore-size polycarbonate filters (Millipore), under low pressure (200 mbar to 1 bar), to collect the total microbial biomass. Similar to the CARD-FISH samples collected in May, June, and July, the water samples were first prefiltered through a 10-µm filter and subsequently through a 3-µm filter before they were filtered through a 0.2-µm filter. Total nucleic acids were isolated according to the protocol of Zhou and colleagues (64). The following modifications to the method were carried out. (i) All centrifugation steps were performed at 53,250 × g, except for the recovery of the aqueous phase after mixing with chloroform-isoamyl alcohol, which was performed at 10,000 × g. (ii) The precipitation of DNA with isopropanol was done overnight at room temperature. (iii) The pellet of crude nucleic acids was resuspended in 1:10 TE buffer (1 mM Tris-HCl and 0.1 mM EDTA [pH 8]). A long time period of DNA precipitation using isopropanol and an increased speed of centrifugation were necessary to retrieve sufficient amounts of DNA.

A fragment of about 1.4 kb in length—encompassing most of the 16S rRNA gene—was amplified using the PLA46F primer (5'-GGA TTA GGC ATG CAA GTC-3') (targeting positions 46 to 63 on the 16S rRNA gene) (39) specific for *Planctomyces* and primer 1392R (5'-ACG GGC GGT GTG TAC-3') (targeting *Escherichia coli* positions 1392 to 1406 on the 16S rRNA gene) (63), a general primer for *Bacteria*. Each PCR mixture with a volume of 50 µl contained 0.3 mg ml<sup>-1</sup> bovine serum albumin (BSA) (Sigma Chemie, Taufkirchen, Germany), 1× reaction buffer (Eppendorf, Hamburg, Germany), 0.25 mM each deoxynucleoside triphosphate (dNTP) (Roche Diagnostics, Mannheim, Germany), 0.5 µM each primer (Biomers, Ulm, Germany), 0.02 U µl<sup>-1</sup> *Taq* polymerase (Eppendorf, Hamburg, Germany) (all concentrations are final concentrations) and 1 µl of DNA. PCR cycling conditions were as follows: an initial denaturation step at 94°C for 4 min; 33 cycles, with 1 cycle consisting of 1 min denaturation at 94°C, 2 min annealing at 59°C, and 1.5 min elongation at 72°C; and a final extension step of 10 min at 72°C. The PCR cycle numbers were reduced as much as possible, and 10 replicates were processed in parallel to minimize PCR biases (43, 52). PCR products were determined with 1% agarose gel electrophoresis (Biozym LE agarose; Biozym, Oldendorf, Germany).

**Cloning, sequencing, and phylogenetic analysis.** To avoid cloning of non-specific and small PCR products, fragments (~1.4 kb) were excised from a 1.5% agarose gel and subsequently extracted and purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) prior to cloning them in pGEM-T Easy (Promega, Madison, WI) according to the manufacturer's instructions. The re-

combinant vectors were then transformed into chemically competent *E. coli* cells (Invitrogen, Gröningen, Netherlands), plated, and picked. Plasmid DNAs were isolated with the Montage plasmid miniprep kit (Millipore, Eschborn, Germany) and then sequenced using the two vector primers M13F (F stands for forward) (5'-GTA AAA CGA CGG CCA G-3') and M13R (R stands for reverse) (5'-CAG GAA ACA GCT ATG AC-3') together with GM1F (5'-CCA GCA GCC GCG GTA AT-3') (29) to obtain almost full-length sequences.

Contigs were assembled with the SEQUENCHER software program version 4.6 (Gene Codes Corporation, Ann Arbor, MI) and aligned in the ARB software package (35) using the SINA\_aligner (44). Alignments were refined manually by comparison to closest relatives. For in-depth phylogenetic rRNA analysis, only sequences longer than 1,200 nucleotides were included. Phylogenetic tree reconstruction was based on distance matrix (DM) (e.g., neighbor-joining) and maximum likelihood (ML) methods with and without 30% and 50% *Planctomyces* positional conservatory filters. Subsequently, a consensus tree was constructed following the standard operating procedure for phylogenetic inference in reference 40.

**Rarefaction analysis.** Diversity coverage was analyzed using the program DOTUR (distance-based operational taxonomic unit and richness) (50). Operational taxonomic units (OTUs) were calculated using the furthest neighbor algorithm for each distance level, from a distance matrix generated in ARB. Then, 97% and 95% sequence identity were selected to generate the rarefaction curves. The coverage index (*C*) of the clone libraries was calculated with the formula  $C = (1 - n/N) \times 100$ , where *N* is the numbers of all clones with a *Planctomyces* insert and *n* is the number of phylotypes at 97% and 95% identity that appeared only once in the library (18, 24).

**Probe design.** Oligonucleotide probes specific for clusters of *Planctomyces* were designed based on the newly created phylogenetic tree using the probe design tool of the ARB software. Probe specificities were checked *in silico* against the SILVA 16S rRNA database (version 100) (44) (see Table S2 in the supplemental material). To optimize the stringency conditions, a series of hybridizations at increasing formamide concentrations (10 to 70%) were evaluated on surface water samples taken at Kabeltonne station. The optimal formamide concentration was the highest concentration before the signal intensity decreased (Table 1).

**Statistical analyses.** All biotic and abiotic parameters were determined by the long-term monitoring program at the Helgoland Biological Station. Multiple linear correlations between *Planctomyces* abundance and abiotic (temperature, salinity, time, dissolved silicate, phosphate, nitrate, nitrite, and ammonium) and biotic (chlorophyll *a* and centric and pennate diatoms) parameters were calculated by the Spearman rank correlation test using SigmaStat 3.5 (Systat Software, Santa Clara, CA). The significance level of the correlations was also adjusted by the Bonferroni correction for multiple comparisons, according to which the *P* value is divided by the total number of simultaneous independent comparisons (30).

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences of this study were deposited in the EMBL Nucleotide Sequence Database under accession numbers FN822075 to FN822228.

## RESULTS AND DISCUSSION

**Temporal trends of *Planctomyces* levels.** The main aim of this study was to assess the diversity, abundance, and spatial distribution of planktonic *Planctomyces* at the coastal marine station Kabeltonne off the Helgoland island in the German Bight of the North Sea. The relative levels of *Planctomyces* at Kabeltonne station were up to 6%. This was higher than in earlier studies at this site (15) and in other marine waters,

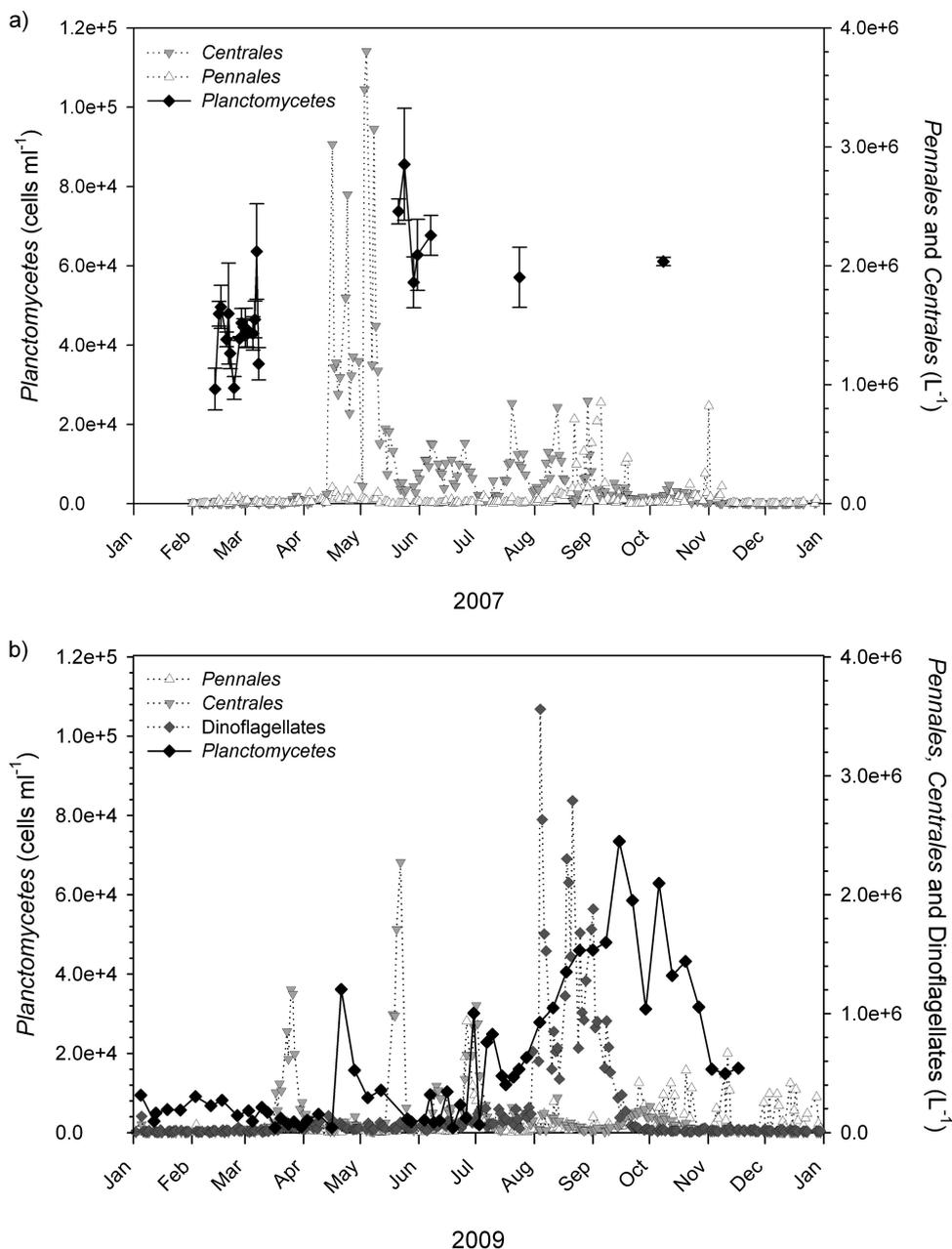


FIG. 2. Absolute counts of *Planctomycetes* (PLA46) and diatoms (*Centrales* and *Pennales*) in 2007 (a) and 2009 (b). For 2009, dinoflagellate counts are also displayed (b). For *Planctomycetes* levels in 2007, means  $\pm$  standard deviations (SD) (error bars) of three replicates are shown.

where they often accounted for  $<0.5\%$  of the prokaryotic picoplankton (47). Most likely the protocol using achromopeptidase to permeabilize the proteinaceous cell walls of *Planctomycetes* in combination with the more sensitive CARD-FISH method resulted in higher fluorescence intensities and better signal-to-noise ratios (41, 61), which together improved the detection efficiency for target cells.

In 2007, the levels of total picoplankton at Kabeltonne station ranged between  $6.3 \times 10^5$  cells ml<sup>-1</sup> in February and  $1.7 \times 10^6$  cells ml<sup>-1</sup> in October (see Table S1 in the supplemental material). The contribution of *Planctomycetes* to the total picoplankton community throughout 2007 was

quite stable with relative levels of 3.7 to 5.9%, but the absolute numbers of cells varied considerably (Fig. 2a). *Planctomycetes* increased from a minimum of  $2.9 \times 10^4$  ml<sup>-1</sup> in February to  $6.4 \times 10^4$  ml<sup>-1</sup> in March, reaching a maximum on May 24 with  $8.6 \times 10^4$  ml<sup>-1</sup> (Fig. 2a). Samples taken later in the year (Fig. 2a) contained between  $5.7 \times 10^4$  and  $6.8 \times 10^4$  ml<sup>-1</sup>. In winter and spring 2009, *Planctomycetes* counts started low ( $3.0 \times 10^3$  to  $9.5 \times 10^3$  ml<sup>-1</sup>). The peak levels at the end of the summer in 2009 ( $7.7 \times 10^4$  cells ml<sup>-1</sup>) and in autumn 2009 ( $6.5 \times 10^4$  cells ml<sup>-1</sup>) (Fig. 2b) were, however, as high as in 2007. These numbers are slightly higher than the  $4 \times 10^4$  ml<sup>-1</sup> of cells of the plan-

tomycetel clade *Pirellula* counted during a diatom bloom off Oregon by Morris and coworkers (37).

**Correlation of *Planctomycetes* counts with chlorophyll *a* and algal counts.** Diatoms of the *Centrales* group were the major phytoplankton in the spring bloom 2007. They showed the lowest value in February ( $3.6 \times 10^2$  liter<sup>-1</sup>) and started to bloom in the middle of April ( $3.0 \times 10^6$  liter<sup>-1</sup>), reaching a maximum at the beginning of May ( $3.8 \times 10^6$  liter<sup>-1</sup>) (Fig. 2a). *Pennales* diatoms began to bloom at the end of August ( $7.1 \times 10^5$  liter<sup>-1</sup>), reaching a peak at the beginning of September ( $8.5 \times 10^5$  liter<sup>-1</sup>), but they also showed a second peak in November ( $8.2 \times 10^5$  liter<sup>-1</sup>) (Fig. 2a). Among various parameters examined, the absolute levels of *Planctomycetes* in 2007 ( $n = 23$ ) were significantly correlated with chlorophyll *a* concentration ( $r_s = 0.51$ ;  $P < 0.05$ ) and with counts of *Centrales* diatoms ( $r_s = 0.54$ ;  $P \leq 0.01$ ). No correlation with *Pennales* was found (see Table S3 in the supplemental material). There was also a strong negative correlation with the concentrations of nitrate ( $r_s = -0.60$ ;  $P \leq 0.01$ ) and nitrite concentrations ( $r_s = -0.56$ ;  $P \leq 0.01$ ). A likely scenario is that the bloom of *Centrales* depleted nitrate and nitrite and formed substrate for the growth of *Planctomycetes*. An attachment of *Planctomycetes* with diatoms had already been reported before by Morris and coworkers (37).

In the year 2009, *Planctomycetes* and three major phytoplankton groups were quantified in higher resolution than in 2007 (Fig. 2b). The spring algal bloom of 2009 resembled the bloom in 2007; it was dominated by *Centrales* and peak values of  $1.2 \times 10^6$  liter<sup>-1</sup> were reached. The level of *Planctomycetes* increased 1 month later, peaking at  $3.6 \times 10^4$  ml<sup>-1</sup>. In summer, several consecutive algal blooms occurred. At the end of June and beginning of July 2009, the *Pennales* and *Centrales* showed a maximum of  $0.9 \times 10^6$  liter<sup>-1</sup> and  $1.1 \times 10^6$  liter<sup>-1</sup>, respectively. During this time, *Planctomycetes* numbers increased to  $3.0 \times 10^4$  ml<sup>-1</sup>. In early August, a bloom of dinoflagellates set in, reaching a peak of  $3.6 \times 10^6$  liter<sup>-1</sup>. *Planctomycetes* continuously increased during August, reaching their annual maximum of  $7.7 \times 10^4$  ml<sup>-1</sup> at the end of the dinoflagellate bloom in mid-September.

Our results are in line with a bottom-up control of *Planctomycetes* by algal production. Cultured marine *Planctomycetes* are heterotrophs specialized for sugar utilization (58, 62). The genome annotation of *Rhodopirellula baltica* SH 1<sup>T</sup> indicated that it can also derive energy from degradation of sulfated polysaccharides of algal origin such as carrageenans and fucans (16, 49). The main storage carbohydrate in marine diatoms is the polysaccharide chrysolaminarin which can contribute up to 80% of the organic carbon (1). While this storage compound is located intracellularly in live algae (5), it may be released, e.g., by algal cell lysis (4). This would explain the phase shifts of algal and *Planctomycetes* maxima of up to several weeks.

**Levels of *Planctomycetes* in size-fractionated samples and macroaggregates.** *Planctomycetes* are well-known for their ability to attach to surfaces by the excretion of holdfast substances (58). Evidence for attachment to marine aggregates had been obtained directly from microscopic observations (37) or indirectly from clone frequencies in small subunit (SSU) rRNA gene libraries (7, 8, 45). In this study, direct microscopic evidence for an attachment could be obtained by CARD-FISH (see Fig. S1b, S1c, and S1f in the supplemental material). On

six sampling days in May (4 days), June (1 day), and July (1 day), water samples were size fractionated (see Fig. S2 in the supplemental material). The average absolute number of *Planctomycetes* cells in the 0.2- to 10- $\mu$ m fraction ( $6.7 \times 10^4$  ml<sup>-1</sup>) was 1 order of magnitude higher than in the 0.2- to 3- $\mu$ m fraction ( $6.5 \times 10^3$  ml<sup>-1</sup>) (see Fig. S2 in the supplemental material). The relative levels of *Planctomycetes* in the 0.2- to 3- $\mu$ m fraction did not exceed 1% of the total picoplankton cells. We calculated that in the late spring and summer of 2007, approximately 90% of *Planctomycetes* resided in the 3- to 10- $\mu$ m fractions, corroborating a clear preference for an attached lifestyle (Fig. S2).

Three macroaggregates were retrieved by scuba diving on 5 June 2007. They were of algal origin, consisting mostly of the red alga *Polysiphonia violacea* and green algae of the genus *Bryopsis*. Relative *Planctomycetes* levels in the 10- to 0.2- $\mu$ m size fraction of sonicated macroaggregates were 6%, 8%, and 10%, further demonstrating the preference of *Planctomycetes* for attachment to algae.

**Diversity of *Planctomycetes*.** Since knowledge on the diversity of *Planctomycetes* in temperate coastal marine waters is still limited, comparative sequence analyses of three 16S rRNA gene libraries were performed as a part of this study. These were constructed with the *Planctomycetes*-specific forward primer PLA46F and the universal primer 1392R, which yield almost full-length 16S rRNA sequences, enabling high-quality phylogenetic reconstructions. A total of 216 sequences were analyzed, of which 184 were affiliated with *Planctomycetes*. Rarefaction analyses indicated that with this number of clones examined, 84 to 92% of the diversity retrieved in the libraries was covered at a level of 97% sequence identity, roughly reflecting species level (see Fig. S3 in the supplemental material). Coverage on an assumed genus level of 95% sequence identity ranged from 89 to 96% (see Fig. S3 in the supplemental material). We compared an unfractionated surface water sample from 23 February 2007, reflecting a late winter situation, with a summer sample taken on 24 July 2007. From the sample collected on 24 July 2007, two libraries were constructed, one of the 3- to 10- $\mu$ m size fraction and one of the 0.2- to 3- $\mu$ m size fraction. Figure 3 shows an overview consensus tree that summarizes the 16S rRNA-based phylogeny of 352 almost full-length sequences (>1,200 nucleotides) of which 154 were obtained in this study, thereby significantly enlarging the availability of high-quality 16S rRNA sequence information on marine *Planctomycetes*. Most of the sequences ( $n = 150$ ) grouped into four distinct phylogenetic clades. These were termed *Pirellula*-related group D, *Pirellula*-related group C, uncultured *Planctomycetes* group B, and *Planctomyces*-related group A. The arrangement of clades A to D remained stable in different types of tree reconstructions (neighbor joining, maximum likelihood) with and without *Planctomycetes* positional conservatory filters (50% and 30%) (Fig. 3). The clades will be discussed in more detail below in the context of specific cell counts.

**Development of a set of oligonucleotide probes specific for marine coastal clades of *Planctomycetes*.** The 16S rRNA sequences of planctomycetel origin retrieved in this study formed the basis for the development of specific oligonucleotide probes. For all four clades, specific oligonucleotide probes were designed with tools of the ARB package (Table 1), tested

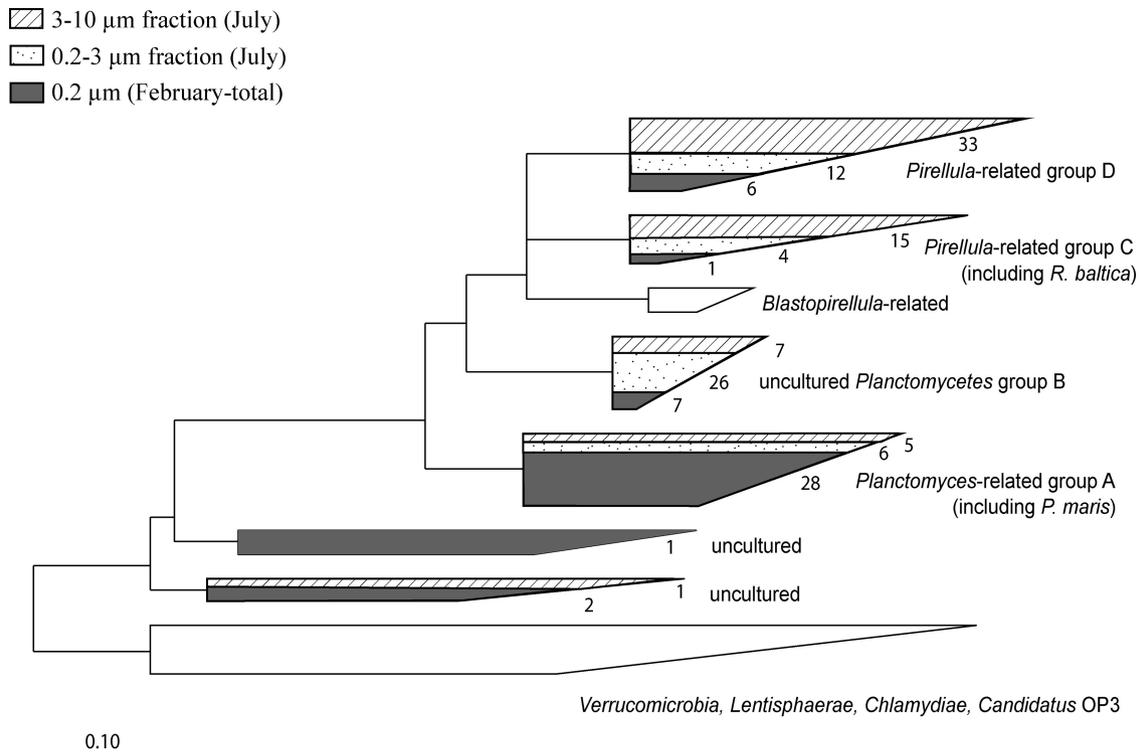


FIG. 3. Phylogenetic tree based on almost full-length 16S rRNA sequences (>1,200 nucleotides) showing the affiliations of different clusters. The consensus tree was built based on maximum likelihood and neighbor-joining trees calculated with and without 50% and 30% *Planctomyces* positional conservatory filters using *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Candidatus* OP3 sequences as the outgroup. Sequences retrieved in this study are indicated by different patterns. The numbers next to the boxes indicate the number of sequences retrieved in this study for each sample. The bar represents 10% sequence divergence.

*in silico* against the curated SILVA-Ref database (44), and subsequently applied to surface water samples taken at Kabeltonne station in 2007 to 2009. The probe designed for the *Pirellula*-related group C did not detect sufficient cells for a statistically robust quantification in any of the samples (data not shown). Therefore, its performance with respect to probe sensitivity and specificity could not be fully evaluated, and we therefore refrain from reporting it.

(i) ***Planctomyces*-related group A.** Twenty-eight of 39 sequences affiliated with *Planctomyces*-related group A were retrieved from the unfractionated February 2007 sample (Fig. 3 and see Fig. S4a in the supplemental material). Three of these sequences were closely related to *Planctomyces maris* (~97% sequence identity), the rest shared <90% sequence identity with *Planctomyces maris* and <89% sequence identity with *Planctomyces brasiliensis*. An oligonucleotide probe was designed to specifically target group A. Probe PlaA1228 (Table 1) targeted 30 of the 39 *Planctomyces*-related group A sequences retrieved in this study (see Fig. S4a in the supplemental material). First, CARD-FISH counts with probe PlaA1228 showed higher numbers in February 2007 ( $1.3 \times 10^4$  cells  $\text{ml}^{-1}$  [1.5%]) and October 2007 ( $3.1 \times 10^4$  cells  $\text{ml}^{-1}$  [1.9%]) than in July 2007 ( $9.0 \times 10^2$  cells  $\text{ml}^{-1}$  [0.1%]) (Fig. 4a). In May and June 2007, no probe-positive cells could be detected. The more finely resolved clade-specific counts from 2009 confirmed higher levels in late autumn and winter. The number of cells that hybridized with PlaA1228 were  $7.1 \times 10^3$   $\text{ml}^{-1}$  (0.5%) and

$9.9 \times 10^3$   $\text{ml}^{-1}$  (0.9%) in October 2009 and  $7.2 \times 10^3$   $\text{ml}^{-1}$  (0.8%) in November 2009 but were below  $1.0 \times 10^3$   $\text{ml}^{-1}$  the rest of the year (Fig. 4b). One can speculate that the members of the *Planctomyces*-related group A are positively influenced by the late autumn bloom of *Pennales* (Fig. 2b). Alternatively, the increased abundance of *Planctomyces*-related group A in winter could also be the result of sediment resuspension by winter storms. This is supported by the fact that sequences of this clade have previously also been retrieved from sediments (see Fig. S4a in the supplemental material). Members of this clade were also detected on the three algal macroaggregates examined with levels of 0.1%, 0.3%, and 0.5%.

(ii) **Uncultured *Planctomyces* group B.** The 16S rRNA gene clone library made from the 0.2- to 3- $\mu\text{m}$  fraction of samples collected in July 2007 was dominated by members of the uncultured *Planctomyces* group B (26 out of 40 sequences), suggesting that most of the cells of this clade are free-living (see Fig. 3). Closely related sequences had been previously retrieved from Oregon coastal water, marine surface waters, and marine sponges (see Fig. S4b in the supplemental material). Probe uPlaB440 was designed to target 32 out of the 40 sequences retrieved in the uncultured *Planctomyces* group B. Between  $3.3 \times 10^3$  cells  $\text{ml}^{-1}$  (0.4%) in February and  $2.3 \times 10^4$  cells  $\text{ml}^{-1}$  (1.4%) in October could be detected in 2007 (Fig. 4a). Since the majority of the uncultured *Planctomyces* group B sequences were retrieved from the 0.2- to 3- $\mu\text{m}$  fraction, this population was quantified in the respective size fractions sam-

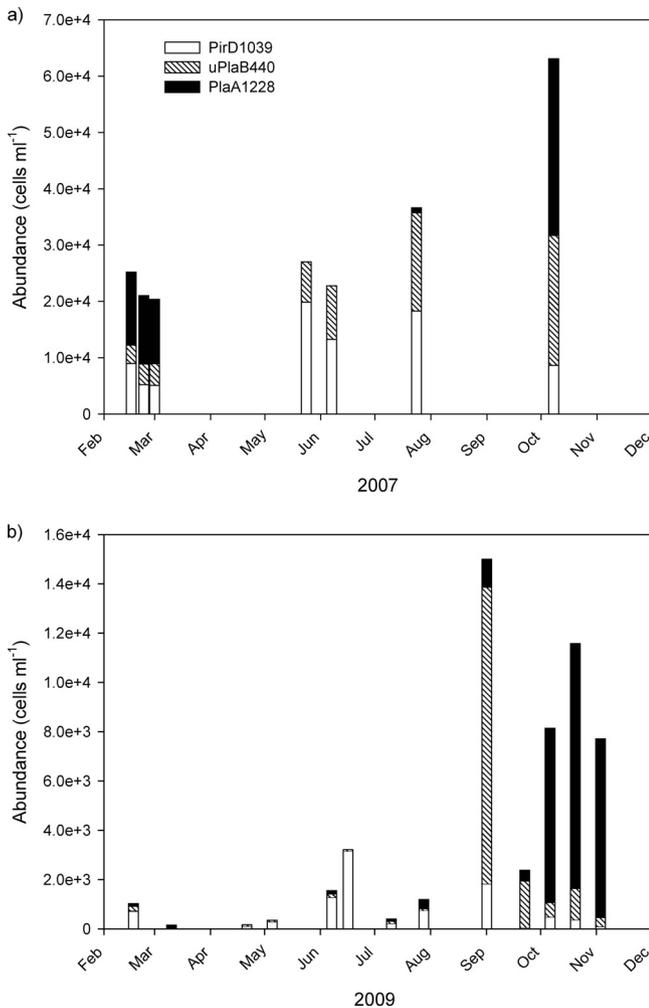


FIG. 4. Clade-specific *Planctomycetes* counts in 2007 (a) and 2009 (b). At least 500 cells (2007) were counted on 15-ml samples from 2007 in three replicate samples. The coefficients of variation ranged between 3% and 66%, except for PlaA1228 in June (173%). In 2009, 100-ml filters were used and at least 20 fields of view were counted. The coefficients of variation were between 30% and 40% for counts above  $\sim 8.0 \times 10^3 \text{ ml}^{-1}$ , but they were higher (up to 300%) for lower counts (also see reference 17). Error bars are not shown for clarity.

pled in May, June, and July 2007. While in July,  $5.1 \times 10^3 \text{ cells ml}^{-1}$  were detected, in May and June, no cells could be detected. However, uPlaB440 counts in the 0.2- to 10- $\mu\text{m}$  fraction in the July 2007 sample were quite high at  $1.7 \times 10^4 \text{ cells ml}^{-1}$ , indicating that this clade also prefers, at least temporarily, an attached lifestyle. On one of the algal macroaggregates, uPlaB440-positive cells could be detected at a low level (0.2%). In 2009, group B showed the highest number in September ( $1.2 \times 10^4 \text{ cells ml}^{-1}$  [0.4%]). Lower levels were observed in other seasons (Fig. 2b). We hypothesize that members of the uncultured *Planctomycetes* group B could be specialized and utilize substrates formed by the late summer dinoflagellate blooms (Fig. 4b), because members of the uncultured *Planctomycetes* group B peaked in autumn in both years (Fig. 4a and b).

(iii) **Pirellula-related group D.** The 3- to 10- $\mu\text{m}$  clone library of the July 2007 water sample was dominated by sequences affiliated with the *Pirellula*-related group D (Fig. 3 and see Fig.

S4c in the supplemental material). The sequences have only low sequence identities with cultured representatives ( $\sim 86\%$  identity with *Rhodopirellula baltica*,  $\sim 88\%$  identity with *Blas-topirellula marina* and  $\sim 89\%$  identity with strains of the genus *Pirellula*). Most of the *Pirellula*-related group D clones originated from the 3- to 10- $\mu\text{m}$  fraction sampled in July 2007, suggesting an attached life style for this clade. Interestingly, the same clade also comprises sequences retrieved from marine sponges and the surfaces of marine macroalga (Fig. S4c).

The newly designed probe PirD1039 targets all of the sequences of the *Pirellula*-related group D. The number of cells hybridizing with PirD1039 ranged from  $5.1 \times 10^3 \text{ ml}^{-1}$  (0.6%) in March 2007 to  $2.0 \times 10^4 \text{ ml}^{-1}$  (1.3%) in May 2007. Generally, higher counts were found in late spring and summer than in autumn and winter in 2007 (Fig. 4a). In 2009, highest counts were obtained with the probe PirD1039 at the beginning and at the end of the summer (e.g.,  $3.2 \times 10^3 \text{ cells ml}^{-1}$  or 0.2% in June 2009) (Fig. 4b). From both years examined, it seems that *Pirellula*-related group D cells are present throughout the year but tend to be slightly more abundant in summer than in winter (Fig. 4a and b). *Pirellula*-related group D cells were detected on all three macroaggregates in levels ranging from 0.6% to 0.8%, which together with the absence of PirD1039-positive cells in the 0.2- to 3- $\mu\text{m}$  fraction of July 2007 supports an attached lifestyle.

**Conclusions.** Our data suggest that the importance of aerobic heterotrophic *Planctomycetes* in coastal marine waters might have been underestimated. At Kabeltonne station, we found levels of up to 6% in the 0.2- to 10- $\mu\text{m}$  fraction, particularly in the context of algal blooms. Future attempts to analyze the *Planctomycetes* should consider their well-documented ability to switch from planktonic life to attachment to particles and potentially also resuspension from sediments. We could show that in late spring and early summer low *Planctomycetes* levels in the  $<3\text{-}\mu\text{m}$  fraction coincide with levels that are 1 order of magnitude higher in the 0.2- to 10- $\mu\text{m}$  fraction. Since in coastal systems particle-attached *Bacteria* can at certain times be as abundant as free-living bacteria, they must be included in ecological studies.

All three clades of marine *Planctomycetes* were present at Kabeltonne station throughout the 2 years examined. Abundance maxima in different seasons indicate a succession of planctomycetal clades. On the basis of the indications for a bottom-up regulation, we speculate that the three dominant clades are partitioned in different niches defined by the utilization of different substrates of algal origin. The successive blooms of *Centrales*, dinoflagellates, and *Pennales* might provide different types of substrates which support the growth of specialized clades of *Planctomycetes*.

Overall, with the clade-specific probes, we were able to detect between 32 and 103% of the *Planctomycetes* community in 2007 and up to 48% of the *Planctomycetes* community in 2009. This new set of probes therefore enables the differentiation of a major fraction of the marine coastal *Planctomycetes*. With the new probes, the hypothesis of a tight coupling of specific clades of *Planctomycetes* with particular groups of algae can be tested at other coastal sites. It should be specifically checked whether the seasonal pattern of a peak of *Pirellula*-related group D at the beginning of the summer followed by peaks of uncultured *Planctomycetes* group B (end of summer/early autumn) and

dominance of *Planctomycetes*-related group A in late autumn and winter is stable in temperate coastal marine settings.

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